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13. ABSTRACT (Maximum 200 Words) Clinical studies have indicated that high breast tumor levels of plasminogen activator inhibitor-1 (PAI-1) are associated with an increased risk for metastasis, decreased patient survival, a well developed angiogenic response, and overall poor prognosis. Since PAI-1 is required for tumor-dependent angiogenesis and inhibits capillary regression, a targeted molecular genetic approach was devised to ablate PAI-1 synthesis in endothelial cells using antisense PAI-1 expression constructs. Work in year 01 established that constructs prepared in the Rc/CMV and retroviral pLNCX2 vectors, in fact, significantly attenuated PAI-1 synthesis in mouse (MS1) and rat (T2) endothelial cells confirming proof of principal for the original hypothesis. Of a total of 11 different clones isolated following transfection of the PAI-1 antisense Rc/CMVIAP plasmid, 4 MS1- and 2 T2-derived lines were essentially PAI-1-null. A host mouse breeding colony was also established in order to maintain, at relatively low cost, a sufficient number of mice to meet the goals of this program.				
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Introduction

Clinical studies have demonstrated that high tumor levels of plasminogen activator inhibitor type-1 (PAI-1) are consistently associated with a markedly increased risk for metastasis, significantly decreased patient survival and an overall poor prognosis (1,2). The role of PAI-1 as a determinant in tumor progression is particularly relevant in the case of breast cancer where elevated PAI-1 expression in the primary breast carcinoma signals an aggressive angiogenic response (3-10). Tumor-initiated angiogenesis requires proteolysis of the endothelial basement membrane, migration of endothelial cells through the extracellular matrix (ECM) toward the angiogenic stimulus and continued endothelial proliferation behind the migrating front (11-13) (Figure 1). Stimulated endothelial cell locomotion requires cycles of ECM adhesion-deadhesion and precise control of the pericellular proteolytic environment (12-14). PAI-1 functions in this process to limit plasmin generation by inhibiting the catalytic activity of urokinase plasminogen activator (uPA) (15,16) modulating, thereby, uPA-dependent ECM degradation in *in vivo* cell motility (17-19). While endothelial cell migration and capillary sprouting requires proteolysis (12,20,21), excessive protease activity prevents the coordinated assembly of endothelial cells into capillary structures highlighting the requirement for an appropriate proteolytic 'balance' for a successful angiogenic response (22,23). Genetic studies *in vivo*, moreover, have implicated PAI-1 as an important regulator of this balance (24,25). Indeed, PAI-1 is expressed specifically in angiogenic 'cords' and migrating endothelial cells as well as in stromal cells in direct contact with the sprouting neovessels but not in the quiescent endothelium (26-28). Most significantly, PAI-1^{-/-} mice are incapable of mounting an angiogenic response either to transplanted tumors or implants of potent angiogenic growth factors (e.g., bFGF) (24,27,29); both tumor-associated angiogenesis and tumor invasiveness were restored by injection of PAI-1-expressing adenovirus (24,27). In the specific context of tumor growth, PAI-1 promotes angiogenesis by inhibition of plasmin proteolysis, thus preserving an appropriate matrix scaffold for endothelial invasion as well as providing critical stability to the primitive tumor neovessels (18,24,27). PAI-1 functions, in fact, to stabilize neovessel structure. Indeed, recent studies have shown that uPA-mediated plasmin generation activates MMP1 and 9 resulting in capillary regression (30). Inhibition of PAI-1 activity with neutralizing antibodies accelerates, whereas exogenous PAI-1 inhibits, capillary regression indicating that endogenous PAI-1 is the major negative regulator of this process (30). Continued PAI-1 expression by the formed capillary structures is required to maintain their stability and, in fact, to prevent regression. Our use of inducible vectors to disrupt PAI-1 synthesis, even in formed capillary structures, will be one novel approach to address the important question of whether PAI-1 targeting can have a therapeutic benefit on existing angiogenic networks. This is an important issue for the treatment of established primary tumors and their developed distant metastases.

Body of Report

Work in our laboratory over the past years has focused on defining molecular controls on PAI-1 gene expression in normal and transformed cells and clarifying the role of the PAI-1 protein on cellular growth and invasive behavior. We have shown that it is possible to

genetically manipulate PAI-1 synthesis in endothelial cells transfected with sense and antisense PAI-1 expression vectors (32,33). We hypothesize that molecular targeting of PAI-1 expression can disrupt both the initial as well as the developed angiogenic response to tumor-derived stimuli and, therefore, represents a therapeutically useful and highly novel approach to breast cancer treatment. We propose that targeted attenuation of PAI-1 expression in the developing neovasculature that develops following implantation of human breast carcinoma cells into immunodeficient mice will inhibit the angiogenic response and limit subsequent tumor growth. We further suggest that human endothelial cells genetically-engineered to express inducible PAI-1 antisense transcripts may 'home' to sites of active tumor-initiated angiogenesis, incorporate into the developing capillary network, and destabilize the tumor vasculature upon inducible ablation of PAI-1 synthesis. We expect that such engineered cells will ultimately serve as a therapeutic resource for inducible anti-angiogenic therapy of human breast cancer.

To achieve these aims, the goals in Task 1 in the originally proposed **Statement of Work** were as follows:

- Task 1.** To assess the effect of retroviral delivery of PAI-1 antisense expression vectors on the angiogenic response to implanted breast carcinomas.
- a. To implant retroviral packaging cells, producing ecotropic retroviruses that express constitutive or inducible "tagged" PAI-1 antisense transcripts, and MDA-MB-231 human breast carcinoma cells into the kidney capsule of immunodeficient mice.
 - b. Confirm vector transduction and transcript expression in the tumor vasculature.
 - c. Assess the ability of constitutive and inducible PAI-1 antisense transcript expression to disrupt the development and maintenance, respectively, of the tumor-dependent angiogenic response and the consequences of PAI-1 expression attenuation on tumor growth.

Based on the data summarized in the **Introduction**, our working hypothesis is that genetically-induced temporal changes in the expression of PAI-1 may influence endothelial cell migration, capillary formation and/or capillary network stability. Efforts in year 01 of this study was devoted to confirmation that the genetic constructs (PAI-1 antisense expression vectors) developed would, in fact, result in attenuated PAI-1 synthesis when transfected into murine endothelial cells as well as when delivered to our established T2 line of rat endothelial cells. Transfection studies established that our selected rat PAI-1 mRNA coding sequence, when cloned in antisense orientation into CMV promoter-driven constructs (i.e., in the Rc/CMV expression vector backbone), effectively attenuated PAI-1 synthesis in both mouse (MS1) and rat (T2) endothelial cells. Transfected cells were selected with neomycin and the levels of *de novo* PAI-1 synthesized assessed by labeling with ³⁵S-methionine and gel electrophoresis. Of a total of 11 different clones selected for analysis, 4 MS1- and 2 T2-derived isolates were essentially PAI-1-null. Such transfection approaches were initially employed to assess the potential usefulness of the PAI-1 coding sequence, identified with our CMV-driven Rc/CMV expression vector, to down-regulate PAI-1 synthesis when introduced into mouse and rat endothelial cells as an antisense retroviral construct in pLNCX2. As was the case with our Rc/CMV-based construct, the pLNCX2 retroviral PAI-1 antisense delivery system similarly was efficient in suppressing

PAI-1 synthesis in selected clonal isolates confirming proof of principal for our overall targeting strategy. Presently, ecotropic retroviral particles are being produced by construct transfection into the EcoPack HEK-293 cell line so that population-wide infection protocols could be evaluated in a manner similar to the that done for selected clonal isolates. An additional significant advance in the year 01 was our establishment of a host mouse breeding colony in the Animal Research Facility at the Albany Medical College that will maintain, at relatively low cost, a sufficient number of mice to meet the goals of this program.

Key Research Accomplishments

It was confirmed that the overall molecular targeting approach and genetic constructs (PAI-1 antisense expression vectors) developed would, in fact, result in attenuate PAI-1 protein synthesis when transfected into murine (MS1) and rat (T2) endothelial cells.

Of a total of 11 different clones selected for analysis, 4 MS1- and 2 T2-derived isolates were essentially PAI-1-null.

Transfection approaches also established the potential usefulness of the PAI-1 coding sequence, identified with our CMV-driven Rc/CMV expression vector, to down-regulate PAI-1 synthesis when introduced into mouse and rat endothelial cells as an antisense retroviral construct in pLNCX2.

A host mouse breeding colony was established in the Animal Research Facility at the Albany Medical College that will maintain, at relatively low cost, a sufficient number of mice to meet the goals of this program.

Reportable Outcomes

All the genetically-engineered MS1 and T2 endothelial cell lines (both transfectants and retrovirally-derived) are to be maintained in the laboratory of the PI. These will be utilized in subsequent "homing" studies to disrupt existent angiogenic networks upon incorporation into the capillary structure. These cells will be made available upon request to members of the scientific community engaged in breast cancer research.

Conclusions

The present work is based largely on our continuing hypothesis that molecular targeting of PAI-1 expression in angiogenic vessels represents a unique gene therapy approach that has the distinct advantages of (1) potential cell-specific construct targeting and (2) a high likelihood of success when directed to established angiogenic "beds". Our laboratory has had considerable experience in the construction and utilization of both sense and antisense PAI-1 expression

vectors (summarized in the **Introduction**) and, more recently, in the design of small molecule inhibitors of PAI-1 function and PAI-1 gene transcription. The use of inducible vectors in the present program, to initiate PAI-1 antisense expression in endothelial cells incorporated into formed capillary structures, constitutes an important approach to address the critical question of whether PAI-1 targeting can have a therapeutic benefit on existing angiogenic networks. We envision that a multifaceted attempt to target PAI-1 gene expression in both breast carcinoma cells (see **Introduction**) and in the collateral tumor vascular network would likely require cell type-specific expression modulation control. The goals described in this funded program, in conjunction with the general scope of work ongoing in the laboratory of the PI, reflect these separate but focused efforts to utilize gene therapy approaches to maximize a positive outcome for the management of human breast cancer.

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